

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
15 March 2001 (15.03.2001)

(10) International Publication Number
WO 01/18055 A1

(51) International Patent Classification: C07K 14/705, 1/14, A61K 38/17, 47/02 (74) Agents: WEBSTER, Thomas, D. et al.; Lilly Corporate Center, Indianapolis, IN 56285 (US).

(21) International Application Number: PCT/US00/20807

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(22) International Filing Date: 31 August 2000 (31.08.2000)

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(25) Filing Language: English

Published:

— With international search report.

(26) Publication Language: English

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(30) Priority Data:
60/153,339 10 September 1999 (10.09.1999) US

(71) Applicant (*for all designated States except US*): ELI LILLY AND COMPANY [US/US]; Lilly Corporate Center, Indianapolis, IN 46285 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): ATKINSON, Paul, Robert [US/US]; 4514 Lakeridge Drive, Indianapolis, IN 46234 (US). TIAN, Yu [CN/US]; 13695 Flintridge Pass, Carmel, IN 46033 (US). WITCHER, Derrick, Ryan [US/US]; 10898 Parrot Court, Fishers, IN 46038 (US).



WO 01/18055 A1

(54) Title: FLINT ANALOG COMPOUNDS AND FORMULATIONS THEREOF

(57) Abstract: The present invention provides novel compounds, which comprise a FLINT analog complexed with a divalent metal cation, pharmaceutical formulations thereof, and methods of using such compounds for treating or preventing diseases related to the FasL/Fas interaction.

FLINT COMPOUNDS AND FORMULATIONS THEREOF

Background of the Invention

The present invention is in the field of human medicine, particularly in the treatment and prevention of disorders that may be associated with the binding of FasL to the Fas receptor. More specifically, the present invention relates to compounds and formulations of a FLINT protein.

A number of tumor necrosis factor receptor proteins ("TNFR proteins") and proteins homologous thereto have been isolated in recent years. They have many potent biological effects and aberrant activity of these proteins has been implicated in a number of disease states.

One such TNFR homologue, reported in July, 1998 (Gentz *et al.*, WO 98/30694), binds the protein FAS Ligand and thereby inhibits the activation of another TNFR homologue, FAS, by FAS Ligand (U.S. Provisional Applications Serial Nos. 60/112,577, 60/112,933, and 60/113,407, filed December 17, 18 and 22, 1998, respectively). This new protein is referred to herein as "FAS Ligand Inhibitory Protein" or "FLINT."

Over activation of FAS by FAS Ligand has been implicated in a number of pathological conditions, including runaway apoptosis (Kondo *et al.*, *Nature Medicine* 3(4):409-413 (1997) and Galle *et al.*, *J. Exp. Med.* 182:1223-1230 (1995)) and inflammatory disease resulting from neutrophil activation (Miwa *et al.*, *Nature Medicine* 4:1287 (1998)).

"Runaway apoptosis" is a level of apoptosis greater than normal or apoptosis occurring at an inappropriate time. Pathological conditions caused by runaway apoptosis include organ failure, for example in the liver, kidneys and pancreas. Inflammatory diseases

-2-

associated with excessive neutrophil activation include, but are not limited to, sepsis, ARDS, SIRS and MODS.

The structural properties of proteins may be affected by divalent cations. For example, aggregation and/or precipitation of proteins, as well as oligomerization, may be induced by divalent cations. Aggregation of proteins can impact the ability to produce, purify, formulate and deliver a protein, for example, as a pharmaceutical product. Moreover, aggregation and/or oligomerization can impact the stability of a protein, for example, in storage. In some instances, a protein's stability can be enhanced if aggregated and/or precipitated prior to, or during storage.

FLINT and analogs thereof, for example, analog R218Q, aggregate and eventually precipitate from solution when exposed to divalent cation. For example, analog R218Q purified by IMAC chromatography and elution in 0.4M imidazole, precipitates from solution (See Example 5, *infra*). These observations suggest that FLINT and analogs thereof interact with divalent cations, such as Ni^{+2} , to cause aggregation and/or precipitation.

As FLINT is potentially a useful therapeutic protein, its purification and formulation are important factors to be worked out on the path to development of a pharmaceutical product. While FLINT is known from prior disclosures (See e.g WO 98/30694 and WO 99/50413), its formulation has not been thoroughly investigated, nor has the impact of divalent cation on the aggregation and/or oligomerization of the protein been sufficiently investigated for purposes of realizing the full therapeutic and pharmaceutical utility.

-3-

The present invention relates to a method for eliminating aggregation and/or precipitation of FLINT and analogs thereof, useful in purifying FLINT and its analogs comprising the removal of divalent cation from a solution 5 or other medium comprising FLINT.

The invention relates further to the purification of FLINT and analogs thereof, from a solution of said FLINT or analog, by immobilized metal ion affinity (IMAC) chromatography, comprising removal of divalent cation from 10 said solution.

The invention relates further to a composition comprising FLINT and a divalent metal cation.

The invention relates further to a method for producing a composition comprising FLINT, or FLINT analog, 15 in association with a divalent cation.

The present invention relates further to a pharmaceutical formulation comprising FLINT, or FLINT analog, in association with a divalent metal cation, and with one or more pharmaceutically acceptable carriers, 20 diluents, or excipients.

Accordingly, the present invention provides a FLINT-divalent cation complex, which comprises FLINT complexed with a divalent metal cation, pharmaceutical formulations thereof, and methods for using such compounds 25 in the treatment and/or prevention of disorders that may be associated with the binding of Fas to FasL, and/or LIGHT to the LT β R and/or TR2/HVEM receptors.

Summary of the Invention

30 The invention provides a composition comprising FLINT complexed with a divalent metal cation. The invention additionally provides parenteral pharmaceutical

formulations comprising the FLINT divalent cation compositions and methods for using such compounds in treating or preventing diseases and disorders that may be associated with the binding of Fas to FasL, and/or LIGHT to 5 LT β R and/or TR2/HVEM receptors. The invention further provides a process of preparing such compounds, which comprises combining FLINT and a divalent metal cation in an aqueous solution at a pH of about 4.5 to 9.0.

10

Detailed Description and Preferred Embodiments

For purposes of the present invention, as disclosed and claimed herein, the following terms and abbreviations are defined as follows:

The term "aggregate" or "aggregation" refers to a 15 non-covalent association of protein or peptide molecules including monomers, subunits, and fragments thereof, that may lead to precipitation of said molecules.

"FLINT" is used herein to encompass native FLINT (SEQ ID NO:2), or mature FLINT (SEQ ID NO:1). The term may 20 also refer to an analog of FLINT having one or more amino acid deletions, substitutions, or additions such that substantially the same biological activity is retained.

The term "fusion protein" or "FLINT fusion protein" as used herein refers to a FLINT protein wherein 25 said protein or analog is fused to a heterologous protein or peptide including, for example, a peptide tag useful in purification, e.g. a His-tag.

"Treating" as used herein, describes the management and care of a patient for the purpose of 30 combating the disease, condition, or disorder and includes the administration of a protein of the present invention to prevent the onset of the symptoms or complications,

-5-

alleviating the symptoms or complications, or eliminating the disease, condition, or disorder.

"Isotonicity agent" refers to an agent that is physiologically tolerated and embarks a suitable tonicity 5 to the formulation to prevent the net flow of water across the cell membrane. Compounds, such as glycerin, are commonly used for such purposes at known concentrations. Other possible isotonicity agents include salts, e.g., NaCl, dextrose, and lactose.

10 The term "oligomer" or "oligomerization" refers to a specific interaction of more than one protein subunit in non-covalent or covalent Fashion. Examples of specific oligomers would include dimers, trimers, tetramers, etc. As used herein the term refers to association of one or more 15 FLINT molecules including association of identical or non-identical subunits such as, for example, native FLINT in association with a FLINT analog or FLINT fusion protein. The process of oligomerization lies on a continuum with the process of aggregation, the latter representing non- 20 specific interactions, that in the extreme, leads to precipitation.

25 "Physiologically tolerated buffer" refers to buffers including TRIS, sodium acetate, sodium phosphate, or sodium citrate. The selection and concentration of buffer is known in the art.

30 "Pharmaceutically acceptable preservative" refers to a multi-use parenteral formulation that meets guidelines for preservative effectiveness to be a commercially viable product. Pharmaceutically acceptable preservatives known in the art as being acceptable in parenteral formulations include: phenol, m-cresol, benzyl alcohol, methylparaben, chlorobutanol, p-cresol, phenylmercuric nitrate, thimerosal

-6-

and various mixtures thereof. Other preservatives may be found, e.g., in Wallhauser, K. H. Develop. Biol. Standard, 24, 9-28 (Basel, S. Krager, 1974). The concentration necessary to achieve preservative effectiveness is
5 dependent upon the preservative used and the conditions of the formulation.

As noted above, the invention provides a compound comprising FLINT protein complexed with a divalent metal cation. A FLINT-divalent cation complex could increase the
10 stability of the protein and enhance potency, as has been observed with other proteins. Such complexes can provide effective pharmacological treatment at lower doses thereby abrogating toxic or other undesirable side effects.

Applicants have discovered that FLINT undergoes
15 oligomerization and/or aggregation in the presence of divalent cations. In one aspect of the present invention, pharmaceutical compositions of FLINT and divalent cation(s) provide depot formulations for therapeutic use. In another aspect, oligomerization and/or aggregation of FLINT can be
20 reduced, prevented, or reversed by removal of divalent cation from said protein, or from the environment in which said protein occurs. In this aspect, the invention relates to a process or method for purifying FLINT and/or for maintaining FLINT in solution. In another aspect, the
25 invention relates to a method to stimulate or induce oligomerization, and/or aggregation of FLINT by the addition of divalent cation to a sample of FLINT.

The presently claimed compounds comprise FLINT complexed with a divalent metal cation. A divalent metal cation includes, for example, Zn^{+2} , Mn^{+2} , Fe^{+2} , Co^{+2} , Cd^{+2} ,
30 Ca^{+2} , Ni^{+2} and the like. A combination of two or more divalent metal cations is operable; however the preferred

compounds comprise a single species of metal cation, most preferably Zn^{+2} . Preferably, the divalent metal cation is in excess; however, the molar ratio of at least one molecule of a divalent metal cation for each ten molecules 5 of FLINT is operable. Preferably, the compounds comprise from 1 to 100 divalent metal cations per molecule of FLINT. The compounds may be amorphous or crystalline solids.

Appropriate forms of metal cations are any form of a divalent metal cation that is available to form a 10 complex with a molecule of FLINT protein of the present invention. The metal cation may be added in solid form or it may be added as a solution. Several different cationic salts can be used in the present invention. Representative examples of metal salts include the acetate, bromide, 15 chloride, fluoride, iodide and sulfate salt forms. The skilled artisan will recognize that there are many other metal salts which also might be used in the production of the compounds of the present invention. Preferably, zinc acetate or zinc chloride is used to create the zinc-FLINT 20 protein compounds of the present invention. Most preferably, the divalent metal cationic salt is zinc chloride.

Generally, the claimed compounds are prepared by techniques known in the art. For example, it is convenient 25 to combine FLINT with the desired divalent metal cation in an aqueous solution at a pH of about 4.5-9.0, preferably about pH 5.5-8, alternatively, pH 6.5-7.6. The claimed compound precipitates from the solution as a crystalline or amorphous solid. Significantly, the compound is easily 30 isolated and purified by conventional separation techniques appreciated in the art, including filtration and centrifugation. Significantly, the protein-metal cation

complex is stable and may be stored as a solid or as an aqueous suspension.

The present invention further provides a pharmaceutical formulation comprising a compound of the 5 present invention and water. The concentration of the FLINT protein in the formulation is about 0.1 mg/mL to about 100 mg/mL; preferably about 0.5 mg/mL to about 50.0 mg/mL; most preferably, about 5.0 mg/mL.

The formulation preferably comprises a 10 pharmaceutically acceptable preservative at a concentration necessary to maintain preservative effectiveness. The relative amounts of preservative necessary to maintain preservative effectiveness varies with the preservative used. Generally, the amount necessary can be found in 15 Wallhauser, K. H. Develop. Biol. Standard, 24, 9-28 (Basel, S. Krager, 1974), herein incorporated by reference.

An isotonicity agent, preferably glycerin, may be added to the formulation. The concentration of the 20 isotonicity agent is in the range known in the art for parenteral formulations, preferably about 16 mg/mL glycerin. The pH of the formulation may also be buffered with a physiologically tolerated buffer. Acceptable physiologically tolerated buffers include TRIS, sodium acetate, sodium phosphate, or sodium citrate. The 25 selection and concentration of buffer is known in the art.

Other additives, such as a pharmaceutically acceptable excipients like Tween 20 (polyoxyethylene (20) sorbitan monolaurate), Tween 40 (polyoxyethylene (20) sorbitan monopalmitate), Tween 80 (polyoxyethylene (20) 30 sorbitan monooleate), Pluronic F68 (polyoxyethylene polyoxypropylene block copolymers), BRIJ 35 (polyoxyethylene (23) lauryl ether), and PEG (polyethylene

glycol) may optionally be added to the formulation to reduce aggregation.

The claimed pharmaceutical formulations are prepared in a manner known in the art, and are administered 5 individually or in combination with other therapeutic agents. The formulations of the present invention can be prepared using conventional dissolution and mixing procedures. Preferably, the claimed formulations are prepared in an aqueous solution suitable for parenteral 10 use. That is, a protein solution is prepared by mixing water for injection, buffer, and a preservative. Divalent metal cations are added to a total cation concentration of about 0.001 to 5.0 mg/mL, preferably 0.05 to 1.5 mg/mL. The pH of the solution may be adjusted to completely 15 precipitate the FLINT protein-cation complex. The compound is easily resuspended before administration to a patient.

Parenteral daily doses of the compound are in the range from about 1 ng to about 10 mg per kg of body weight, although lower or higher dosages may be administered. The 20 required dosage will be determined by the physician and will depend on the severity of the condition of the patient and upon such criteria as the patient's height, weight, sex, age, and medical history.

Variations of this process would be recognized by 25 one of ordinary skill in the art. For example, the order in which the components are added, whether a surfactant is used, the temperature and pH at which the formulation is prepared, may all be optimized for concentration and means of administration.

30 The pH of the formulation is generally pH 4.5 to 9.0 and preferably 5.5 to 8.0, alternatively 6.5 to 7.6;

-10-

although more acidic pH wherein a portion or all of the protein-metal cation complex is in solution is operable.

The formulations prepared in accordance with the present invention may be used in a syringe, injector, pumps 5 or any other device recognized in the art for parenteral administration.

The proteins used in the present compounds can be prepared by any of a variety of recognized peptide synthesis techniques including classical (solution) 10 methods, solid phase methods, semi synthetic methods, and more recent recombinant DNA methods. Recombinant methods are preferred if a high yield is desired. The basic steps in the recombinant production of protein include:

- a) construction of a synthetic or semi-synthetic (or isolation from natural sources) DNA encoding the FLINT protein,
- b) integrating the coding sequence into an expression vector in a manner suitable for the expression of the protein either alone 20 or as a fusion protein,
- c) transforming an appropriate eukaryotic or prokaryotic host cell with the expression vector, and
- d) recovering and purifying the recombinantly 25 produced protein.

Synthetic genes and nucleic acids can be constructed by techniques well known in the art. Owing to the degeneracy of the genetic code, the skilled artisan 30 will recognize that multiple DNA sequences may be constructed which encode the desired proteins. Synthesis is achieved by recombinant DNA technology or by chemical

-11-

synthesis, for example, see Brown, et al. (1979) Methods in Enzymology, Academic Press, N.Y., Vol. 68, pgs. 109-151. A DNA sequence encoding FLINT can be generated using a conventional DNA synthesizing apparatus such as the Applied Biosystems Model 380A or 380B DNA synthesizers (commercially available from Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404). It may be desirable in some applications to modify the coding sequence of the FLINT protein so as to incorporate a convenient protease sensitive cleavage site, e.g., between the signal peptide and the structural protein facilitating the controlled excision of the signal peptide from the fusion protein construct.

A gene encoding FLINT may also be created by using the polymerase chain reaction (PCR) using suitable primers designed in accordance with SEQ ID NO:3 (FLINT cDNA). The template can be a cDNA library from a tissue that expresses FLINT, for example (commercially available from Clonetech or Stratagene). Such methods are well known in the art, *c.f.* Maniatis, et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989) herein incorporated by reference.

The constructed or isolated DNA sequences are useful for expressing FLINT either by direct expression or as a fusion protein. When the sequences comprise a fusion gene construct, release of FLINT will require enzymatic or chemical cleavage from the fusion partner. A variety of peptidases which cleave a polypeptide at specific sites or digest the peptides from the amino or carboxy termini (e.g. diaminopeptidase) of the peptide chain are known. Furthermore, particular chemicals (e.g. cyanogen bromide)

-12-

will cleave a polypeptide chain at specific sites. The skilled artisan will appreciate the modifications necessary to the amino acid sequence (and synthetic or semi-synthetic coding sequence if recombinant means are employed) to 5 incorporate site-specific internal cleavage sites. See U.S. Patent No. 5,126,249; Carter P., Site Specific Proteolysis of Fusion Proteins, Ch. 13 in Protein Purification: From Molecular Mechanisms to Large Scale Processes, American Chemical Soc., Washington, D.C. (1990).

10 Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required.

15 In general, plasmid vectors containing promoters and control sequences which are derived from species compatible with the host cell are used with these hosts. The vector ordinarily carries a replication origin and one or more sequences for selection of transformed cells.

20 The desired coding sequence is inserted into an expression vector in the proper orientation to be transcribed from a promoter and ribosome binding site, both of which should be functional in the host cell in which the protein is to be expressed.

25 In general, prokaryotes are used for cloning of DNA sequences in constructing the vectors useful in the invention. For example, E. coli K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include E. coli B and E. coli X1776 (ATCC 30 No. 31537). These examples are illustrative rather than limiting.

-13-

The DNA molecules may also be recombinantly expressed in eukaryotic expression systems. Preferred promoters for mammalian host cells may be obtained from various sources, for example, the genomes of viruses such 5 as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. •-actin promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction 10 fragment which also contains the SV40 viral origin of replication. Fiers, et al., Nature, 273:113 (1978). The entire SV40 genome may be obtained from plasmid pRSV, ATCC 45019. The immediate early promoter of the human 15 cytomegalovirus may be obtained from plasmid pCMBb (ATCC 77177). Of course, promoters from the host cell or related species also are useful herein.

Transcription of the DNA by higher eucaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually 20 about 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively oriented and positioned independently and have been found 5' (Laimins, L. et al., PNAS 78:993 (1981)) and 3' (Lusky, M. L., et al., Mol. Cell Bio. 3:1108 (1983)) to the transcription 25 unit, within an intron (Banerji, J. L. et al., Cell 33:729 (1983)) as well as within the coding sequence itself (Osborne, T. F., et al., Mol. Cell Bio. 4:1293 (1984)). Many enhancer sequences are now known from mammalian genes 30 (globin, RSV, SV40, EMC, elastase, albumin, alpha-fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 late enhancer, the cytomegalovirus early promoter

enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated 5 cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding protein. The 3' untranslated 10 regions also include transcription termination sites.

Expression vectors may contain a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR, which may be derived from the BglII/HindIII restriction fragment 15 of pJOD-10 [ATCC 68815]), thymidine kinase (herpes simplex virus thymidine kinase is contained on the BamHI fragment of vP-5 clone [ATCC 2028]) or neomycin (G418) resistance genes (obtainable from pNN414 yeast artificial chromosome vector [ATCC 37682]). When such selectable markers are 20 successfully transferred into a mammalian host cell, the transfected mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell 25 line which lacks the ability to grow without a supplemented media. Two examples are: CHO DHFR⁻ cells (ATCC CRL-9096) and mouse LTK⁻ cells (L-M(TK-) ATCC CCL-2.3). These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these 30 cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented

-15-

media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR 5 or TK gene will not be capable of survival in nonsupplemented media.

A suitable vector for eucaryotic expression is pRc/CMV. pRc/CMV is commercially available from Invitrogen Corporation, 3985 Sorrento Valley Blvd., San Diego, CA 10 92121. To confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform E. coli K12 strain DH10B (ATCC 31446) and successful transformants selected by antibiotic resistance where appropriate. Plasmids from the transformants are prepared, 15 analyzed by restriction and/or sequence by the method of Messing, et al., Nucleic Acids Res. 9:309 (1981).

Host cells may be transformed with the expression vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing 20 expression from a promoter, selecting transformants, or amplifying genes. Suitable culture conditions, temperature, pH and the like, are readily apparent to the ordinarily skilled artisan. The techniques of transforming cells with the aforementioned vectors are well known in the art and may be found in such general references as 25 Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989), or Current Protocols in Molecular Biology (1989) and supplements.

30 Suitable host cells for expressing the vectors encoding the claimed proteins in higher eucaryotes include: African green monkey kidney line cell line transformed by

-16-

SV40 (COS-7, ATCC CRL-1651); transformed human primary embryonal kidney cell line 293, (Graham, F. L. *et al.*, *J. Gen Virol.* 36:59-72 (1977), *Virology* 77:319-329, *Virology* 86:10-21); baby hamster kidney cells (BHK-21(C-13), ATCC 5 CCL-10, *Virology* 16:147 (1962)); Chinese hamster ovary cells CHO-DHFR⁻ (ATCC CRL-9096), mouse Sertoli cells (TM4, ATCC CRL-1715, *Biol. Reprod.* 23:243-250 (1980)); African green monkey kidney cells (VERO 76, ATCC CRL-1587); human cervical epitheloid carcinoma cells (HeLa, ATCC CCL-2); 10 canine kidney cells (MDCK, ATCC CCL-34); buffalo rat liver cells (BRL 3A, ATCC CRL-1442); human diploid lung cells (WI-38, ATCC CCL-75); human hepatocellular carcinoma cells (Hep G2, ATCC HB-8065); and mouse mammary tumor cells (MMT 060562, ATCC CCL51).

15 In addition, unicellular eukaryotes such as yeast may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used eukaryotic microorganism, although a number of other strains are commonly available. For expression in *Saccharomyces*, the 20 plasmid YRp7, for example, (ATCC-40053, Stinchcomb, *et al.*, *Nature* 282:39 (1979); Kingsman *et al.*, *Gene* 7:141 (1979); Tschemper *et al.*, *Gene* 10:157 (1980)) is commonly used. This plasmid already contains the trp gene which provides a selection marker for a mutant strain of yeast lacking the 25 ability to grow in tryptophan, for example ATCC no. 44076 or PEP4-1 (Jones, *Genetics* 85:12 (1977)).

Suitable promoter sequences for expression in yeast include the promoters for 3-phosphoglycerate kinase (found on plasmid pAP12BD ATCC 53231 and described in U.S. 30 Patent No. 4,935,350, June 19, 1990) or other glycolytic enzymes such as enolase (found on plasmid pAC1 ATCC 39532), glyceraldehyde-3-phosphate dehydrogenase (derived from

-17-

plasmid pHcGAPC1 ATCC 57090, 57091), *Zymomonas mobilis* (U.S. Patent No. 5,000,000 issued March 19, 1991), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, 5 pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which contain inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions 10 for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein (contained on plasmid vector pCL28XhoLHPV ATCC 39475, U. S. Patent No. 4,840,896), glyceraldehyde 3-phosphate dehydrogenase, and enzymes 15 responsible for maltose and galactose (GAL1 found on plasmid pRY121 ATCC 37658) utilization. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., European Patent Publication No. 73,657A. Yeast enhancers such as the UAS 20 Gal from *Saccharomyces cerevisiae* (found in conjunction with the CYC1 promoter on plasmid YEpsec--hI1beta ATCC 67024), also are advantageously used with yeast promoters.

The following examples are provided to further illustrate the preparation of the formulations of the 25 invention. The scope of the invention is not construed as merely consisting of the following examples.

EXAMPLE 1

Preparation of FLINT-Zinc Formulations

30 About 20 mg of FLINT is completely dissolved in 32 mL of an aqueous solution containing 16 mg/mL glycerin and 2 mg/mL phenol and passed through a sterile 0.2 μ

-18-

filter. An aqueous solution containing 100 mg/mL of zinc in water is prepared from zinc chloride. Dilutions are made to prepare 10 mg/mL zinc and 1 mg/mL zinc solutions. Five 6-mL aliquots of the FLINT solution is mixed with the 5 zinc solutions as shown in Table I:

Table I

Sample	mL of 1 mg/mL zinc added	ML of 10 mg/mL zinc added	ML of 100 mg/mL zinc added	ml of H ₂ O added	Total mg/mL zinc concentration
A	0	0	0	100	0
B	17	0	0	83	0.0027
C	0	33	0	67	0.054
D	0	0	19	81	0.30
E	0	0	92	8	1.50

Each formulation is adjusted to pH 7.48 ± 0.03 using small 10 volumes of 2N and 5N sodium hydroxide and stored at 4°C. Sample A is completely clear while samples B through E are cloudy suspensions.

EXAMPLE 2

15 Analysis of Zinc Formulations

Size-exclusion chromatography is performed on the centrifuged supernatants of Samples A through E of Example 1. For these analyses, 100 uL of the supernatants are injected onto an analytical Superdex-75® (3.2/30, 20 Pharmacia) column equilibrated in PBS (Dulbecco's Phosphate-Buffered Saline, GibcoBRL). The column is eluted at ambient temperature at 0.5 mL/min and the protein in the eluant monitored at 214 nm.

-19-

EXAMPLE 3

Biological Activity of the Zinc Formulations

A FLINT bioassay measuring cell survival (i.e. prevention of apoptosis) is performed in a 96 well plate format with reactions of 100 µl/well. 25 µl of Jurkat cells (5×10^4 cells/well) is mixed with 25 µl of recombinant human FasL (final concentration 150ng/ml) and 50 µl of FLINT samples in Example 1. Cells are incubated at 37°C over night. Twenty µl of MTS tetrazolium compound (U.S. Pat. No. 5,185,450 assigned to the Univ. of South Florida and exclusively licensed to Promega Corporation, Madison, WI) is added to each well and the incubation carried out for 2h at 37°C. Absorbance at 490 nm is recorded using a plate reader.

EXAMPLE 4

Large Scale FLINT Polypeptide Purification

Large scale production of FLINT was carried out by first growing stable clones stable pIG1-FLINT-containing AV12 RGT 18 cells in several 10 liter spinners. After reaching confluence, cells were further incubated for 2-3 more days to secrete maximum amount of FLINT into media. Media containing FLINT was adjusted to 0.1 % CHAPS and concentrated in an Amicon ProFlux M12 tangential filtration system to 350 ml. The concentrated media was adjusted to pH 6.0 and passed over a SP Sepharose Fast Flow (Pharmacia, 50 ml) at a flow rate of 3 ml/min. The column was washed with buffer A (20 mM MOPS, 0.1 % CHAPS, pH 6.0) until the absorbance (280 nm) returned to baseline and the bound polypeptides were eluted with a linear gradient from 0.1 M-0.3 M NaCl (in buffer A) developed over 40 min. followed by a linear gradient from 0.3 to 0.5 M NaCl (in buffer A) developed over 40 min. Fractions containing FLINT were

-20-

pooled and passed over a 1 cm x 15 cm Vydac C4 column equilibrated with 0.1 % TFA/H₂O. The bound FLINT was eluted with a linear gradient 10 % to 35 % CH₃CN/0.1 % TFA over 30 min. followed by a linear gradient from 35 % to 50 %
5 CH₃CN/0.1 % TFA over 20 min. Fractions containing FLINT were pooled, concentrated under vacuum to approximately 2 ml and applied to a Superdex 75 (Hi Load 16/60, Pharmacia) size exclusion column equilibrated with PBS (1 mM potassium phosphate, 3 mM sodium phosphate), 0.5 M NaCl, 10 % glycerol, pH 7.4 at a flow rate of 1.0 ml/min. Fractions
10 containing FLINT were analyzed by SDS-PAGE and found to be greater than 95 % pure. The N-terminal sequence of FLINT was confirmed on the purified polypeptide.

15

EXAMPLE 5

IMAC Purification of R218Q from 293 EBNA Cells

FLINT analog R218Q was purified by IMAC chromatography from 3.4 liters of cell culture medium harvested from 293 EBNA cells that expressed R218Q. The
20 FLINT analog was eluted from the IMAC column in a buffer containing PBS, 0.5 M NaCl, 0.4 M imidazole, pH 7.4. The eluted material was dialyzed against a buffer containing PBS, 0.5 M NaCl, 10% glycerol, pH 7.4. The dialyzed solution turned cloudy suggesting that the protein had precipitated.
25 This was confirmed experimentally by intrinsic tryptophan fluorescence.

This problem was resolved by resuspending the precipitated protein in PBS, 0.5 M NaCl, 10% glycerol, pH 7.4 and adding EDTA to a final concentration of 50 mM. The
30 precipitated protein went back into solution as observed by visual observation and confirmed by typtophan fluorescense.

EXAMPLE 6

Effect of Divalent Cation on FLINT

35

FLINT was purified from either AV12 or 293 cell lines. Protein samples were stored in PBS at pH 7.4, 0.5 M

-21-

NaCl, and 10% glycerol. The effect of divalent cations, such as Ni²⁺, Zn²⁺ and Ca²⁺, was investigated using intrinsic tryptophan fluorescence intensity and fluorescence anisotropy. Since fluorescence anisotropy is very
5 sensitive to the rotational correlation time of the molecule, the change in the value of anisotropy reflects change in the association of FLINT molecules upon addition of divalent cations.

Concentrations of FLINT were measured on an AVIV
10 model 14DS spectrometer. Spectra were collected from 400 nm to 260 nm at 1-nm bandwidth and were corrected for the solvent and scatter using data obtained between 360 nm to 320 nm by the AVIV computer program Loggen. The peak absorbance at about 280 nm was divided by 0.786 mg⁻¹ cm⁻¹ to
15 determine the concentration of the protein in a 1-cm pathlength cell. 5 mM NiCl₂ or ZnCl₂ or CaCl₂ stock solution was made by dissolving in H₂O the appropriate amount of solid NiCl₂, ZnCl₂, or CaCl₂.

20 Tryptophan fluorescence intensity and fluorescence anisotropy were measured using an ISS PCI photon counting spectrofluorometer. A protein solution of about 0.1 mg/ml concentration was excited at 295 nm and the total intensity of fluorescence and fluorescence anisotropy was recorded using a 335 nm cutoff filter in a cell of 5 mm
25 x 10 mm pathlength with a 8 nm excitation bandwidth. A small aliquot of 5 mM NiCl₂, ZnCl₂, or CaCl₂ stock was added to the protein sample in the cell to adjust the concentration of divalent cation concentration. The sample was then mixed by inverting the cuvette after each addition
30 of divalent metal. The fluorescence signal intensity and anisotropy were determined as a function of divalent ion concentration.

-22-

The fluorescence intensity and anisotropy data obtained on FLINT as a function of NiCl_2 or ZnCl_2 is shown in Table I. Addition of either NiCl_2 or ZnCl_2 decreased the fluorescence intensity and increased the anisotropy,
5 indicating an association of FLINT molecules. The association of FLINT molecules upon addition of ZnCl_2 is reversible by addition of 2 mM EDTA, as indicated by the decrease of anisotropy to the initial anisotropy value in the absence of ZnCl_2 .

10

Table I. Typtophan fluorescence intensity and anisotropy of FLINT as a function of NiCl_2 or ZnCl_2 concentration.

[NiCl_2] uM	Intensity	Anisotropy	[ZnCl_2] uM	Anisotropy
0	669978	0.1357	0	0.1344
5	648081	0.14	5	0.1304
10	628453	0.1368	10	0.1273
20	578457	0.1396	20	0.1342
40	548057	0.1416	40	0.1462
80	514699	0.1461	80	0.1724
100	457960	0.1527	100	0.1884
200	443962	0.1578	200	0.2172
400	361070	0.1671	400	0.2436
+ 2 mM EDTA	442029	0.1528	2 mM EDTA	0.1338

15

This example shows that divalent cations, such as Ni^{2+} , Zn^{2+} , and Ca^{2+} , interact with FLINT to cause association of FLINT molecules. Zn^{2+} causes reversible precipitation and can be used in the purification of His-tagged FLINT.

20

-23-

EXAMPLE 6

Treatment of Acute Liver Failure by a Complex of Divalent Cation and FLINT

Using mice, a model of liver damage is induced
5 using a modification of the methods set out in Tsuji H., et al, 1997, Infection and Immunity, 65(5):1892-1898. FLINT is made according to Example 4. Briefly, BALB/c mice (Harlan) per each experimental group are given intravenous injections (the lateral tail vein) of 6 mg of D(+)-
10 Galactosamine (Sigma, 39F-0539) in 100 µl of PBS (GIBCO-BRL) and 3 µg of Lipopolysaccharide B *E.coli* 026:B6 (LPS) (Difco, 3920-25-2) in 100 µl of PBS. The LPS is administered, via i.v. injection, 5 minutes after the galactosamine, which was administered i.v. After LPS
15 challenge, the animals are injected intraperitoneally with FLINT (200 µg), or FLINT complexed with Zinc (as in Example 1). The survival rates of the mice are determined 24 and 48 hours after LPS injection.

When 400 ug of FLINT was administered i.p. to
20 mice, 2 hours before challenge with LPS and GalN, 100% of the animals were alive 48 hours after LPS/GalN dosing (See Figure 6 WO 99/50413). In contrast, only 20% of those animals not treated with FLINT survived 48 hours. It is expected that the Zinc-FLINT formulation would be more
25 potent than non-divalent formulated FLINT.

EXAMPLE 7

Treatment of Cerebral Ischemia by a Complex of Divalent Cation and FLINT

30 Adult male gerbils (70 to 80 g body weight, Charles River Laboratories, Wilmington, MA) are anesthetized by i.p. injections of sodium pentobarbital

-24-

(Nembutal) 40 mg/kg, and additional i.p. injections of 10 mg/kg when necessary to maintain a surgical plane of anesthesia. Animals are placed on a thermostatically controlled heating blanket to maintain body temperature at 5 37 °C. The ventral surface of the neck is exposed, the fur shaved, and the skin cleaned with 2% iodine solution.

After the pre-surgical preparation, a midline incision is made, and the skin opened. The sternohyoid muscles are divided to expose and isolate the common 10 carotid arteries (CCA) for clamping. Sterilized aneurysm clips (blade with 0.15 mm, closing force ~10 gm) are secured by means of a sterilized clip applier on both left and right CCA for 5 minutes. The clamps are then removed and the patency of the arteries checked visually. The 15 wound in the neck is closed by surgical suture.

Immediately following the cerebral ischemia procedure and while the gerbil is still unconscious, the fur on the dorsal surface of the head is shaved and the skin cleaned with 2% iodine solution. Under surgical 20 anesthesia, the gerbil's head is secured in a stable position by means of a stereotaxic apparatus (SA) and a midline incision is made to expose the skull. At a position 1 mm lateral and 1 mm posterior to the bregma, as guided by the vernier scale of the SA, the skull is thinned 25 by a dental drill equipped with a drill bit of 0.5 mm in diameter. The thinned area is punctured with a microsyringe equipped with a 27-gauge blunt needle inserted 3 mm deep for a bolus injection of 5 ul (0.63 mg/ml)of FLINT in phosphate buffer saline (PBS) or zinc-FLINT 30 complex of Example 1.

After the bolus injection, the syringe needle is exchanged for an infusion cannula [3 mm in length] of a

-25-

brain infusion assembly connected to an Alzet osmotic pump (Alza Corp., Palo Alto, CA) which reservoir is placed under the skin on the shoulder of the gerbil. The infusion cannula is anchored on the surface of the skull using
5 dental cement. The wound is closed by surgical suture. The Alzet osmotic pump containing FLINT solution (0.63 mg/ml) or zinc-FLINT is delivered continuously at a rate of 1 μ l/h for 3 days. Gerbils are allowed to survive for 5 days (the surgery day is taken as day zero).

10 On the fifth day of survival, the gerbils are sacrificed in a CO₂ chamber. Thoracotomy is performed for transcardiac perfusion of saline for 3 minutes and formaldehyde for 2 minutes. The brains are removed for histological processing following a standard procedure
15 commonly adapted in the field. Coronal sections are obtained at approximately 1.7 mm posterior to the bregma. After staining with Cresyl violet, the sections are viewed under a microscope at 40x magnification for cell counter quantification of the intact hippocampal neurons along the
20 dorsal CA1 regions (0.5 mm in length) of both hemispheres. Data are analyzed by Student t-Test and the Wilcox ranking test.

Results show that FLINT has a significant effect on neuronal survival compared to vehicle (p=0.0039 in t-
25 Test; p=0.0037 in Wilcoxon Rank Sums) and is indistinguishable from normal controls. Zinc-FLINT complex would be expected to have enhanced potency.

-26-

What is claimed is:

1. A composition comprising a divalent metal cation and FLINT protein.

5

2. A composition as in claim 1 wherein said cation is selected from the group consisting of Zn^{+2} , Ca^{+2} , Ni^{+2} , Mn^{+2} , Fe^{+2} , Co^{+2} , and Cd^{+2} .

10 3. A composition of Claim 1, wherein the divalent metal cation is Zn^{+2} .

15 4. A pharmaceutical formulation comprising a composition of claim 1 in combination with one or more pharmaceutically acceptable carriers, diluents, or excipients.

5. A formulation of Claim 4, wherein the total cation concentration is 0.001 to 5.0 mg/mL.

20 6. A formulation of Claim 5, wherein the total cation concentration is 0.05 to 1.5 mg/mL.

7. A process for reducing aggregation of FLINT molecules comprising the step of removing divalent metal cation.

25

8. A process as in claim 7 wherein said FLINT molecules are in solution.

30 9. A process as in claim 7 wherein said cation is removed by the addition of EDTA.

10. A method for inducing oligomerization of FLINT molecules comprising the step of adding divalent metal cation.

-27-

11. A method for inducing aggregation of FLINT molecules comprising the step of adding divalent metal cation until said molecules precipitate.

SEQUENCE LISTING "

<110> Witcher, Derrick
Tian, Yu
Atkinson, Paul

<120> FLINT Compounds and Formulations Thereof

<130> X-13196

<140>
<141>

<160> 3

<170> PatentIn Ver. 2.0

<210> 1
<211> 271
<212> PRT
<213> Homo sapiens

<400> 1

Val Ala Glu Thr Pro Thr Tyr Pro Trp Arg Asp Ala Glu Thr Gly Glu
1 5 10 15

Arg Leu Val Cys Ala Gln Cys Pro Pro Gly Thr Phe Val Gln Arg Pro
20 25 30

Cys Arg Arg Asp Ser Pro Thr Thr Cys Gly Pro Cys Pro Pro Arg His
35 40 45

Tyr Thr Gln Phe Trp Asn Tyr Leu Glu Arg Cys Arg Tyr Cys Asn Val
50 55 60

Leu Cys Gly Glu Arg Glu Glu Ala Arg Ala Cys His Ala Thr His
65 70 75 80

Asn Arg Ala Cys Arg Cys Arg Thr Gly Phe Phe Ala His Ala Gly Phe
85 90 95

Cys Leu Glu His Ala Ser Cys Pro Pro Gly Ala Gly Val Ile Ala Pro
100 105 110

Gly Thr Pro Ser Gln Asn Thr Gln Cys Gln Pro Cys Pro Pro Gly Thr
115 120 125

Phe Ser Ala Ser Ser Ser Ser Glu Gln Cys Gln Pro His Arg Asn
130 135 140

Cys Thr Ala Leu Gly Leu Ala Leu Asn Val Pro Gly Ser Ser Ser His
145 150 155 160

Asp Thr Leu Cys Thr Ser Cys Thr Gly Phe Pro Leu Ser Thr Arg Val
165 170 175

Pro Gly Ala Glu Glu Cys Glu Arg Ala Val Ile Asp Phe Val Ala Phe
180 185 190

Gln Asp Ile Ser Ile Lys Arg Leu Gln Arg Leu Leu Gln Ala Leu Glu
195 200 205

Ala Pro Glu Gly Trp Gly Pro Thr Pro Arg Ala Gly Arg Ala Ala Leu
210 215 220

Gln Leu Lys Leu Arg Arg Arg Leu Thr Glu Leu Leu Gly Ala Gln Asp
225 230 235 240

Gly Ala Leu Leu Val Arg Leu Leu Gln Ala Leu Arg Val Ala Arg Met
245 250 255

Pro Gly Leu Glu Arg Ser Val Arg Glu Arg Phe Leu Pro Val His
260 265 270

<210> 2

<211> 300

<212> PRT

<213> Homo sapiens

<400> 3

Met Arg Ala Leu Glu Gly Pro Gly Leu Ser Leu Leu Cys Leu Val Leu
1 5 10 15

Ala Leu Pro Ala Leu Leu Pro Val Pro Ala Val Arg Gly Val Ala Glu
20 25 30

Thr Pro Thr Tyr Pro Trp Arg Asp Ala Glu Thr Gly Glu Arg Leu Val
35 40 45

Cys Ala Gln Cys Pro Pro Gly Thr Phe Val Gln Arg Pro Cys Arg Arg
50 55 60

Asp Ser Pro Thr Thr Cys Gly Pro Cys Pro Pro Arg His Tyr Thr Gln
65 70 75 80

Phe Trp Asn Tyr Leu Glu Arg Cys Arg Tyr Cys Asn Val Leu Cys Gly
85 90 95

Glu Arg Glu Glu Ala Arg Ala Cys His Ala Thr His Asn Arg Ala
100 105 110

Cys Arg Cys Arg Thr Gly Phe Phe Ala His Ala Gly Phe Cys Leu Glu
115 120 125

His Ala Ser Cys Pro Pro Gly Ala Gly Val Ile Ala Pro Gly Thr Pro
130 135 140

Ser Gln Asn Thr Gln Cys Gln Pro Cys Pro Pro Gly Thr Phe Ser Ala
145 150 155 160

Ser Ser Ser Ser Glu Gln Cys Gln Pro His Arg Asn Cys Thr Ala
165 170 175

Leu Gly Leu Ala Leu Asn Val Pro Gly Ser Ser Ser His Asp Thr Leu
180 185 190

Cys Thr Ser Cys Thr Gly Phe Pro Leu Ser Thr Arg Val Pro Gly Ala

195	200	205
Glu Glu Cys Glu Arg Ala Val Ile Asp Phe Val Ala Phe Gln Asp Ile		
210	215	220
Ser Ile Lys Arg Leu Gln Arg Leu Leu Gln Ala Leu Glu Ala Pro Glu		
225	230	235
Gly Trp Gly Pro Thr Pro Arg Ala Gly Arg Ala Ala Leu Gln Leu Lys		
245	250	255
Leu Arg Arg Arg Leu Thr Glu Leu Leu Gly Ala Gln Asp Gly Ala Leu		
260	265	270
Leu Val Arg Leu Leu Gln Ala Leu Arg Val Ala Arg Met Pro Gly Leu		
275	280	285
Glu Arg Ser Val Arg Glu Arg Phe Leu Pro Val His		
290	295	300

<210> 3
<211> 936
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (25)...(924)

<p><400> 3</p> <p>gctctccctg ctccagcaag gacc atg agg gcg ctg gag ggg cca ggc ctg Met Arg Ala Leu Glu Gly Pro Gly Leu 1 5</p> <p>tcg ctg ctg tgc gtg ttg gcg ctg cct gcc ctg ccg gtg ccg Ser Leu Leu Cys Leu Val Leu Ala Leu Pro Ala Leu Leu Pro Val Pro 10 15 20 25</p> <p>gct gta cgc gga gtg gca gaa aca ccc acc tac ccc tgg cg gac gca Ala Val Arg Gly Val Ala Glu Thr Pro Thr Tyr Pro Trp Arg Asp Ala 30 35 40</p> <p>gag aca ggg gag cgg ctg gtg tgc gcc cag tgc ccc cca ggc acc ttt Glu Thr Gly Glu Arg Leu Val Cys Ala Gln Cys Pro Pro Gly Thr Phe 45 50 55</p> <p>gtg cag cgg ccg tgc cgc cga gac agc ccc acg acg tgt ggc ccg tgt Val Gln Arg Pro Cys Arg Arg Asp Ser Pro Thr Thr Cys Gly Pro Cys 60 65 70</p> <p>cca ccg cgc cac tac acg cag ttc tgg aac tac ctg gag cgc tgc cgc Pro Pro Arg His Tyr Thr Gln Phe Trp Asn Tyr Leu Glu Arg Cys Arg 75 80 85</p> <p>tac tgc aac gtc ctc tgc ggg gag cgt gag gag gag gca cgg gct tgc Tyr Cys Asn Val Leu Cys Gly Glu Arg Glu Glu Ala Arg Ala Cys 90 95 100 105</p>	<p>51</p> <p>99</p> <p>147</p> <p>195</p> <p>243</p> <p>291</p> <p>339</p>
---	--

cac gcc acc cac aac cgt gcc tgc cgc tgc cgc acc ggc ttc ttc gcg His Ala Thr His Asn Arg Ala Cys Arg Cys Arg Thr Gly Phe Phe Ala	110	115	120	387
cac gct ggt ttc tgc ttg gag cac gca tcg tgt cca cct ggt gcc ggc His Ala Gly Phe Cys Leu Glu His Ala Ser Cys Pro Pro Gly Ala Gly	125	130	135	435
gtg att gcc ccg ggc acc ccc agc cag aac acg cag tgc cag ccg tgc Val Ile Ala Pro Gly Thr Pro Ser Gin Asn Thr Gln Cys Gln Pro Cys	140	145	150	483
ccc cca ggc acc ttc tca gcc agc agc tcc agc tca gag cag tgc cag Pro Pro Gly Thr Phe Ser Ala Ser Ser Ser Ser Glu Gln Cys Gln	155	160	165	531
ccc cac cgc aac tgc acg gcc ctg ggc ctg gcc ctc att gtg cca ggc Pro His Arg Asn Cys Thr Ala Leu Gly Leu Ala Leu Ile Val Pro Gly	170	175	180	185
tct tcc tcc cat gac acc ctg tgc acc agc tgc act ggc ttc ccc ctc Ser Ser Ser His Asp Thr Leu Cys Thr Ser Cys Thr Gly Phe Pro Leu	190	195	200	627
agc acc agg gta cca gga gct gag gag tgt gag cgt gcc gtc atc gac Ser Thr Arg Val Pro Gly Ala Glu Glu Cys Glu Arg Ala Val Ile Asp	205	210	215	675
ttt gtg gct ttc cag gac atc tcc atc aag agg ctg cag cgg ctg ctg Phe Val Ala Phe Gln Asp Ile Ser Ile Lys Arg Leu Gln Arg Leu Leu	220	225	230	723
cag gcc ctc gag gcc ccg gag ggc tgg gct ccg aca cca agg ggc ggc Gln Ala Leu Glu Ala Pro Glu Gly Trp Ala Pro Thr Pro Arg Ala Gly	235	240	245	771
cgc gcg gcc ttg cag ctg aag ctg cgt cgg cgg ctc acg gag ctc ctg Arg Ala Ala Leu Gln Leu Lys Leu Arg Arg Leu Thr Glu Leu Leu	250	255	260	265
ggg gcg cag gac ggg gcg ctg ctg gtg cgg ctg ctg cag ggc ctg cgc Gly Ala Gln Asp Gly Ala Leu Leu Val Arg Leu Leu Gln Ala Leu Arg	270	275	280	867
gtg gcc agg atg ccc ggg ctg gag cgg agc gtc cgt gag cgc ttc ctc Val Ala Arg Met Pro Gly Leu Glu Arg Ser Val Arg Glu Arg Phe Leu	285	290	295	915
cct gtg cac tgatcctggc cc Pro Val His				936
		300		

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
15 March 2001 (15.03.2001)

PCT

(10) International Publication Number
WO 01/18041 A3

(51) International Patent Classification²: C07K 14/705, A61K 38/17

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(21) International Application Number: PCT/US00/20805

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(22) International Filing Date: 31 August 2000 (31.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/153,445 10 September 1999 (10.09.1999) US

(71) Applicant (*for all designated States except US*): **ELI LILLY AND COMPANY** [US/US]; Lilly Corporate Center, Indianapolis, IN 46285 (US).

Published:

— *With international search report.*

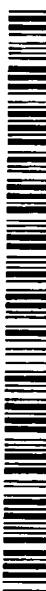
(88) Date of publication of the international search report:
14 June 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **ATKINSON, Paul, Robert** [US/US]; 4514 Lakeridge Drive, Indianapolis, IN 46234 (US). **TIAN, Yu** [CN/US]; 13695 Flintridge Pass, Carmel, IN 46033 (US). **WITCHER, Derrick, Ryan** [US/US]; 10898 Parrot Court, Fishers, IN 46038 (US).

(74) Agents: **WEBSTER, Thomas, D.** et al.; Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285 (US).



WO 01/18041 A3

(54) Title: FLINT PROTEINS AND FORMULATIONS THEREOF

(57) Abstract: The present invention provides novel compounds, which comprise FLINT complexed with a divalent metal cation, pharmaceutical formulations thereof, methods for reducing and/or inducing aggregation of FLINT, and methods of using such compounds for treating or preventing diseases that may be related to the FasL/Fas interaction.

INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/US 00/20805

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/705 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ, CHEM ABS Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	<p>CARPENTER KATHARINE A ET AL: "Aggregation behaviour and Zn²⁺ binding properties of secretin." BIOCHEMISTRY, vol. 37, no. 48, 1 December 1998 (1998-12-01), pages 16967-16974, XP002161821 ISSN: 0006-2960</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex

* Special categories of cited documents

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *I* document which may throw doubts on priority, claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

& document member of the same patent family

Date of the actual completion of the international search

7 March 2001

Date of mailing of the international search report

20/03/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5618 Patentlaan 2
NL - 2280 HV Rijswijk
Tel (+31-70) 340-2040, Tx 31 651 epo nl.
Fax (+31-70) 340-3016

Authorized officer

Cervigni, S

INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/US 00/20805

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate of the relevant passages	Relevant to claim No.
A	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1996 ESLER WILLIAM P ET AL: "Zinc-induced aggregation of human and rat beta-amyloid peptides in vitro." Database accession no. PREV199698674161 XP002161822 abstract & JOURNAL OF NEUROCHEMISTRY, vol. 66, no. 2, 1996, pages 723-732, ISSN: 0022-3042</p> <p>---</p> <p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1995 RICCIO P ET AL: "Specificity of zinc binding to myelin basic protein." Database accession no. PREV199598520931 XP002161823 abstract & NEUROCHEMICAL RESEARCH, vol. 20, no. 9, 1995, pages 1107-1113, ISSN: 0364-3190</p> <p>---</p> <p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1991 PERMYAKOV E A ET AL: "BINDING OF ZINC-II IONS TO ALPHA LACTALBUMIN" Database accession no. PREV199293060793 XP002161824 abstract & JOURNAL OF PROTEIN CHEMISTRY, vol. 10, no. 6, 1991, pages 577-584, ISSN: 0277-8033</p> <p>---</p> <p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1986 JEFFREY P D: "SELF-ASSOCIATION OF DES-B-26-B-30 INSULIN THE EFFECT OF CALCIUM AND SOME OTHER DIVALENT CATIONS" Database accession no. PREV198682043196 XP002161825 abstract & BIOLOGICAL CHEMISTRY HOPPE-SEYLER. vol. 367, no. 5, 1986. pages 363-370, ISSN: 0177-3593</p> <p>---</p> <p>WO 99 50413 A (MIZRAHI JACQUES ;HEUER JOSEF GEORG (US); NOBLITT TIMOTHY WAYNE (US) 7 October 1999 (1999-10-07)</p> <p>---</p>	
P,A		-/-

INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/US 00/20805

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document with indication where appropriate of the relevant passages	Relevant to claim No
A	EP 0 861 850 A (SMITHKLINE BEECHAM CORP) 2 September 1998 (1998-09-02) ---	
A	WO 98 30694 A (HUMAN GENOME SCIENCES INC ;FENG PING (US); NI JIAN (US); EBNER REI) 16 July 1998 (1998-07-16) cited in the application ----	
A	WO 99 14330 A (GENENTECH INC) 25 March 1999 (1999-03-25) -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l Application No

PCT/US 00/20805

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9950413	A	07-10-1999	AU	3369199 A	18-10-1999
			BR	9909328 A	12-12-2000
			NO	20004873 A	24-11-2000
			AU	2211100 A	12-07-2000
			WO	0037094 A	29-06-2000
EP 0861850	A	02-09-1998	US	5885800 A	23-03-1999
			CA	2220852 A	03-08-1998
			JP	10215886 A	18-08-1998
WO 9830694	A	16-07-1998	AU	729062 B	25-01-2001
			AU	5815798 A	03-08-1998
			AU	6238698 A	03-08-1998
			BR	9806954 A	21-03-2000
			CN	1247567 T	15-03-2000
			EP	1007659 A	14-06-2000
			EP	0990031 A	05-04-2000
			WO	9830693 A	16-07-1998
WO 9914330	A	25-03-1999	AU	9497098 A	05-04-1999
			EP	1015587 A	05-07-2000